EXHIBIT 11
A189 – A192

AMYLOSE (Blue Value)

PRINCIPLE

Iodine complexes preferentially with the amylose (linear fraction) in corn starch. The starch sample (defatting is optional) is dispersed in alkali, and neutralized. An excess of standard iodine solution is added, and the resulting stable blue complex is measured spectrophotometrically. Amylose content is estimated by reference to a calibration plot, prepared with the aid of defatted starches having known amylose contents (Note 1).

SCOPE

This method is applicable to unmodified corn starches.

SPECIAL APPARATUS

Spectrophotometer: An instrument having a continuously-variable wavelength control in the visible region, equipped with a red-sensitive detector, and with matching 1.0-cm cuvets is recommended.

REAGENTS

1. Iodine Solution, 0.20%: Dissolve 20.0 g of reagent grade potassium iodide (KI) in 200 ml of distilled water in a 1-liter volumetric flask. Add 2.000 g of resublimed iodine (I_2) and stir. When iodine is dissolved completely, dilute to volume with distilled water, and mix. Store in a nonactinic bottle.

   Iodine solution prepared in this manner will contain exactly 2.00 mg iodine per ml, and it is stable for long periods. Standardize the solution occasionally against National Bureau of Standards arsenious oxide. When iodine concentration falls below 1.98 mg per ml, discard and prepare fresh reagent.
2. Sodium Hydroxide Solution, 1 *N*: Standard
3. Hydrochloric Acid Solution, 0.1 *N*: Standard
4. Ethyl Alcohol: Absolute
5. Phenolphthalein Indicator, 0.1%

AMYLOSE (Blue Value)—*continued*

PROCEDURE

Sample Analysis: Grind sample to eliminate hard or coa se particles, blend, and determine moisture content by an approved method. Defatted samples usually provide more accurate data, and defatting, if desired, can be accomplished rapidly and efficiently by extracting with a mixed solvent composed of methanol and dimethylsulfoxide (Note 2).

Weigh a starch sample containing 100.0 ± 0.1 mg of dry substance and transfer quantitatively to a 100-ml volumetric flask. Add 1 ml of ethyl alcohol and swirl to disperse the starch. Add 10.0 ml of 1 *N* sodium hydroxide solution and again swirl to disperse sample. Allow dispersion to stand until sample is completely gelatinized (about 1 hour). The mixture must be smooth and free of lumps. Dilute to volume with distilled water and mix.

Pipet 2.50 ml of this solution into a 100-ml volumetric flask, and add 50 ml of distilled water. Add 2 drops of phenolphthalein indicator and titrate with 0.1 *N* hydrochloric acid solution from a buret until the pink indicator color just disappears. Add 2.0 ml of 0.20% iodine solution, dilute to volume with distilled water, mix, and let stand 30 minutes.

Prepare a reference solution (blank) by diluting 2.0 ml of 0.20% iodine solution to 100 ml volume with distilled water, and mix before use. Fill one of two 1-cm matching cuvets with the reference solution and fill the other with the sample solution. Determine the transmittance of sample solution at 620 nm against the reference solution.

Standardization: Calibration data are obtained by similar analysis of defatted, unmodified starches. Amylose contents of the starches used for standardization should range from 0% (waxy maize) to 75% (high-amylose maize). If the standard starches are not defatted, defat by the rapid extraction procedure using the methanol-dimethylsulfoxide mixed solvent (Note 2), or defat by extracting with 85% methanol as described in CRA Standard Analytical Method B-28. If the amylose contents are unknown, iodine affinities are determined and amylose contents are calculated using the methods outlined in CRA Standard Analytical Method B-28.

AMYLOSE (Blue Value)—*continued*

Process the defatted starch standards by the same procedure described under "Sample Analysis." Determine transmittance (T) of the standard sample solutions against the reference solution at 620 nm in 1.0-cm matching cuvets. Plot transmittance (T) versus amylose content, on semi-logarithmic graph paper for the starch standards.

CALCULATION

Defatted Samples:

Amylose Content, % = % Amylose (From Graph)

Nondefatted Samples (Note 3):

Amylose Content, % = % Amylose (From Graph)× 1.06

NOTES AND PRECAUTIONS

1. This method is similar to that described by G. A. Gilbert and S. P. Spragg, Methods in Carbohydrate Chemistry, Vol. 4, p. 168 (R. L. Whistler, Ed., Academic Press, New York 1964).
2. Samples and standard starches may be defatted rapidly by the following procedure. Place 10 to 11 g of starch sample in a 250-ml Erlenmeyer flask equipped with a T 24/40 joint. Insert a stirring bar, add 30 ml of anhydrous methanol and 20 ml of anhydrous dimethylsulfoxide. Place flask in heating mantle supported on a magnetic stirrer, and attach a water-cooled condenser. Stir at a moderate rate; bring sample-solvent mixture to reflux temperature, and reflux for 30 minutes.

   Transfer hot mixture to medium-porosity, sintered-glass Buchner funnel and recover starch by vacuum filtration. Rinse flask into funnel with anhydrous methanol from a wash bottle, and wash starch with an additional 50 ml of anhydrous methanol; maintain vacuum until excess solvent is removed.

   Transfer starch cake to a 125-ml Erlenmeyer flask, and insert a magnetic stirring bar. Add 50 ml of anhydrous methanol and heat to boiling on a steam

AMYLOSE (Blue Value)—*continued*

bath. Remove from steam bath, stir on a magnetic stirrer for 15 minutes, and again heat to boiling on a steam bath. As before, filter the hot mixture on a medium-porosity, sintered-glass Buchner funnel, recovering the starch by vacuum filtration. Wash the starch with 50 ml of hot anhydrous methanol, maintain vacuum until excess solvent is removed.

Transfer extracted starch sample to a watch glass or Petri dish, and air-dry overnight at room temperature.

3. The correction factor, 1.06, was developed empirically for nondefatted starch. It is recommended that laboratories wishing to apply the above method to nondefatted samples should develop their own correction factor.

EXHIBIT 12
A193 – A203

Cereal Chemistry

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1970

OLIPIDS ON LOAF VOLUME
FLOUR AND 1 g. GERM

oaf Volume of Bread Baked with Following Phospholipid Levels		
1.0% cc.	1.5% cc.	2.0% cc.
81	78	79
72	76	76
83	86	88
71	70	72
81	84	86
74	72	78
86	88	90

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Amylose Determination in Dimethyl Sulfoxide Extracts of Maize[1]

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ABSTRACT

About 1 g. of corn is ground in 90% dimethyl sulfoxide (9:1 DMSO-water, v./v.) and then shaken for 24 hr. at room temperature to dissolve most of the starch. After undissolved solids are removed, the starch in 10 ml. of the clear extract is purified by precipitation with ethanol. The DMSO-starch precipitate is redissolved in 50 ml. of 90% DMSO. Starch in this solution is determined polarimetrically in an electronic polarimeter ($[\alpha]^{25°}_{546\ m\mu}$ = 220°). Amylose is determined spectrophotometrically on a separate portion of the same solution by measuring the absorbance of the amylose-iodine complex at 615 mμ. Purified linear starch fractions and waxy starch serve as reference standards for calibration.

Amylomaize starches solubilize more readily in 90% DMSO than do either ordinary or waxy starches. However, the method is applicable to determination of amylose in the entire range of corn from ordinary corn to amylomaize. DMSO-extractable substances of germ and pericarp do not interfere with determination of amylose in endosperm starch. In fractional extraction of corn with DMSO, initial amylose content is high and decreases in successive extracts. Selective precipitation of starch with ethanol from crude DMSO extracts is equivalent to conventional solvent extraction as a means of minimizing fatty-acid interference with iodine sorption. Rate of amylose analysis is one and one-half to two determinations per man-hour.

[1]Presented at the 54th annual meeting, Chicago, April-May 1969. Contribution from the Northern Regional Research Laboratory, Peoria, Illinois 61604. This is a laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

[2]Biometrical Services, Agricultural Research Service, U.S. Department of Agriculture, stationed at Peoria.

Development of corn having starch with a high amylose content has been the objective of an intensified breeding program in which the Northern Laboratory has co-operated. Interest in high-amylose corn, which has been given the name "amylomaize," stems from properties of the linear molecular component of the starch—amylose. The potential usefulness of high-amylose starch and the properties of amylose have been discussed previously (1,2,3).

To report percent amylose in starch, it is necessary to determine first the starch content and secondly the concentration of amylose. In the procedure now used, starch is determined polarimetrically in a partially purified dimethyl sulfoxide (DMSO) extract of corn. Determination of amylose on a separate portion of the same extract is based on measuring spectrophotometrically the blue color of the amylose-iodine complex (4,5).

The procedure is similar in general approach to that of Shuman and Plunkett (6), except that these workers dissolved the starch with a hot, concentrated calcium chloride solution at a pH of 2.0. Under these conditions, starch degrades considerably.

DMSO was chosen rather than calcium chloride because it had been shown by Foster and co-workers (7,8) that DMSO was an effective starch solvent that apparently did not alter the dissolved starch chemically. Furthermore, solutions of starch are stable in the solvent for long periods (9,10).

Flexibility of the procedure for amylose determination is enhanced because starch is stable in DMSO. Corn may be extracted with the solvent, either at room temperature or more rapidly at higher temperature, without significant difference in amylose results. Samples which cannot be analyzed immediately may be steeped in DMSO until it is convenient to proceed with the analysis. The solvent penetrates readily into endosperm cells, swells the starch granules, and facilitates subsequent grinding and extraction. When kept in the dark, the amylose-iodine complex is stable for more than 24 hr. in the presence of the small volume of DMSO (0.5 to 1.0 ml. in 100 ml. of aqueous solution) introduced with the starch sample.

McGuire and Erlander (11) used DMSO as a solvent to isolate starch from corn rather than to determine amylose. Shannon (12) extracted starch primarily from immature maize by exhaustive extraction with hot (70°C.) DMSO and determined total carbohydrate in the extract.

Our procedure was developed primarily to determine amylose rather than total starch. Lipids, proteins, soluble sugars, and polysaccharides (such as hemicelluloses) which occur in corn do not interfere with determination of amylose by this method. A simple procedure is followed, based on analysis of about 1-g. samples of corn in which extraction with DMSO and all other operations are carried out at room temperature. The method lends itself to analysis of large numbers of samples as required for genetic studies. Output per man-hour is one and one-half to two amylose analyses.

MATERIALS AND METHODS

Samples analyzed included varieties of corn ranging from waxy maize to amylomaize. Corn with ordinary starch included commercial yellow dent and flour corns. Most of the samples analyzed were amylomaize containing starch with

generally more than 50% apparent amylose. These were mostly selections submitted by corn breeders; however, numerous analyses were made of commercial amylomaize (Amicorn 5 and 7).

DMSO, industrial grade purchased in drum lots, was the solvent. It was diluted with distilled water to 90% DMSO (v./v.). This ratio of DMSO to water is within the optimal range for solubilizing starch (9).

Determination of Starch

Starch, dissolved in 90% DMSO, was determined polarimetrically. Optical rotation of the solution was measured with an electronic polarimeter equipped with a digital readout (Bendix automatic polarimeter, Model No. 143). Measurements were made in green light (interference filter transmitting 5,461 A ±15 A; half-band width, 100 A ±20 A). Optical rotation of starch solutions was read in a glass flow-through cell of 5-cm. path length. Concentration of starch, c, in the solution was calculated from equation 1:

$$c = \frac{100\,a}{[220^\circ]\,P_{Pol}} \qquad (1)$$

where a = observed angular rotation, degrees;

$[220^\circ]$ = specific rotation of starch in 90% DMSO in green light, 546 mμ;

P_{Pol} = path length of polarimeter cell, decimeters.

The polarimeter was calibrated against U.S. Bureau of Standards sucrose solution accurately made up in distilled water to contain about 1 g. of sucrose per 100 ml. of solution.

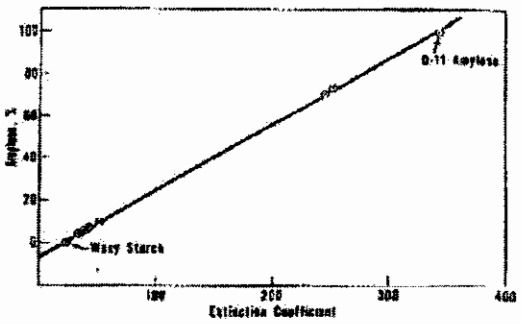
Determination of Amylose

Amylose content[3] (13) of the starch was determined spectrophotometrically by measuring absorbance of the amylose-iodine complex. The following reagents were used:

Hydrochloric acid, 0.5N	2.0 ml.
Potassium iodide, 0.016N	5.0 ml.
Potassium iodate, 0.0051N	5.0 ml.

To prepare the blue starch-iodine solution, about 50 ml. of distilled water was added to a 100-ml. volumetric flask. Five milliliters each of potassium iodide and potassium iodate was then added and an accurately measured aliquot (0.5 or 1.0 ml.) of the DMSO-starch solution was delivered into the flask with a calibrated Seligson pipet. Finally 2.0 ml. of 0.5N hydrochloric acid was added. The volume

[3] The term "amylose" as used here is equivalent to the term "apparent amylose" (13); the latter presupposes that a part of the iodine is sorbed by long side chains on the branched starch component. If this condition exists, amylose values of starches should be somewhat high on the basis of measurement of their total iodine sorption.



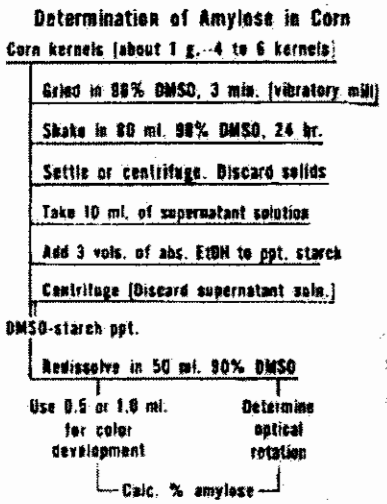


Fig. 1 (left). Calibration curve, showing linear relation between amylose concentration of starch and the extinction coefficient.

Fig. 2 (right). Steps followed in analyzing amylose in corn.

was adjusted to 100 ml. with distilled water.

The absorbance of this solution was read on a Beckman B Model spectrophotometer at 615 mμ.

Reference Standards

The linear relation between the absorbance of amylose-iodine solutions and the concentration of amylose is shown in Fig. 1, and the equation for the curve is given under "Calculations" (eq. 2). Iodine affinity of reference standards, expressed as percent amylose, was plotted against the extinction coefficient ($E^{1\%}_{1\ cm.}$). Reference standards, all of known iodine titer, included a purified corn amylose, a waxy corn starch, and intermediate amyloses and amylopectins from corn. Iodine affinity was determined by the method of Bates et al. (14) as modified by Wilson et al. (15). The standards were dissolved in 90% DMSO, and 0.5- or 1.0-ml. aliquots were used to prepare 100 ml. of amylose-iodine solution as described under "Determination of Amylose."

The basic reference standard was a purified corn amylose, designated D-11, prepared from Iowa hybrid 939 corn starch by fractionation with butanol by the Schoch procedure (16). After purification by four successive precipitations from butanol, the final product had an iodine sorptive capacity of 200 mg. of iodine per g. This product was taken as 100% amylose. The wave length of peak absorption for D-11 amylose-iodine complex was 636 mμ; the extinction coefficient at this wave length, $E^{1\%}_{1\ cm.}$, was approximately 365.

Absorption maxima of starches ranging in amylose content from normal to high amylose varied between 593 and 625 mμ with an average value of 615 mμ. This

mean wave length was adopted for measurement of absorbance of all corn starches analyzed.

The waxy reference starch was obtained from Iowa 939 waxy corn. This starch was assumed to be 100% amylopectin (i.e., 0% amylose). The extinction coefficient of this waxy starch was about 26 at 615 mμ.

General Procedure

The scheme of analysis is outlined in Fig. 2.

About 1.1 g. of corn (representing about 1 g. of endosperm) is soaked overnight in distilled water in the refrigerator at 7°C. The entire sample may be ground or the germ and pericarp may be removed and only the endosperm processed. The sample is ground for 3 min. with 40 ml. of 90% DMSO on a vibratory mill (React-R-Mill, Udy Analyzer Co., Boulder, Colo.) in a nickel grinding chamber. The chamber is rinsed twice with 20-ml. portions of 90% DMSO.

Washings and ground sample are combined and shaken continuously for 24 hr. at room temperature. After the samples are removed from the shaker, cell walls, protein, and other cellular debris may be separated either by centrifugation or by gravity sedimentation.

Ten milliliters of the clear supernatant is added slowly, with constant agitation, to 30 ml. of ethanol in a polyethylene centrifuge tube, fitted with a polyethylene screw cap. The mixture is shaken for 2 hr. and the precipitate of DMSO-starch is centrifuged. The supernatant mixture of DMSO and alcohol is drained and the tube is inverted over paper toweling. Fifty milliliters of 90% DMSO is added and the centrifugate is dissolved on a continuous shaker. Either 0.5 or 1.0 ml. of this purified solution is taken for colorimetric estimation of amylose; optical rotations are measured on a separate portion of the same solution.

Calculations

The basic equation relating percent amylose and the absorbance of the blue amylose-iodine solution is:

$$\text{Percent amylose} = 0.296 \frac{A}{c_1 P_A} - 7.67 \qquad (2)$$

where A = absorbance of amylose-iodine solution;
P_A = path length of colorimeter cell, cm.;
c_1 = concentration of starch in the blue solution, g./100 ml.

Starch concentration, c, is determined polarimetrically in the purified DMSO-starch solution as shown in eq. 1. The final starch concentration, c_1, in the colored solution was derived from c by applying a dilution correction (eq. 3) in preparing 100 ml. of aqueous colored solution with a small volume (0.5 or 1.0 ml.) of DMSO-starch solution.

$$c_1 = \frac{c}{F_D} \qquad (3)$$

where F_D = dilution factor. For example, if 0.5 ml. of DMSO-starch solution is diluted to 100 ml., F_D has a value of 200.

Percent amylose is calculated by combining spectrophotometric and polarimetric data:

$$\text{Percent amylose} = \frac{0.296\ A[220^\circ]P_{Pol}F_D}{100\ a\ P_A} - 7.67 \qquad (4)$$

RESULTS AND DISCUSSION

Specific Rotation

An accurate value is required for the specific rotation of starch in DMSO at 546 mμ.

Specific rotation ($[\alpha]^{25^\circ}_{546\ m\mu}$) of a series of purified starches and starch fractions was determined in 90% DMSO. The values for $[\alpha]^{25^\circ}_{546\ m\mu}$ ranged from about +219° to +222° with an average of +220.48 ± 0.79 for a series of 13 starches and starch fractions. There are no significant differences in specific rotation between starches, amyloses, or amylopectins. Apparently alpha (1→6) branch points of amylopectins have no detectable effect on the main alpha (1→4)-linked chain. These results confirm earlier work of Neely (17), who likewise found no significant difference in specific rotation between amylose and amylopectin. Neely reported a value of +175°C. for the specific rotation of starch $[\alpha]_D$ in DMSO; he did not measure the rotation at 546 mμ.

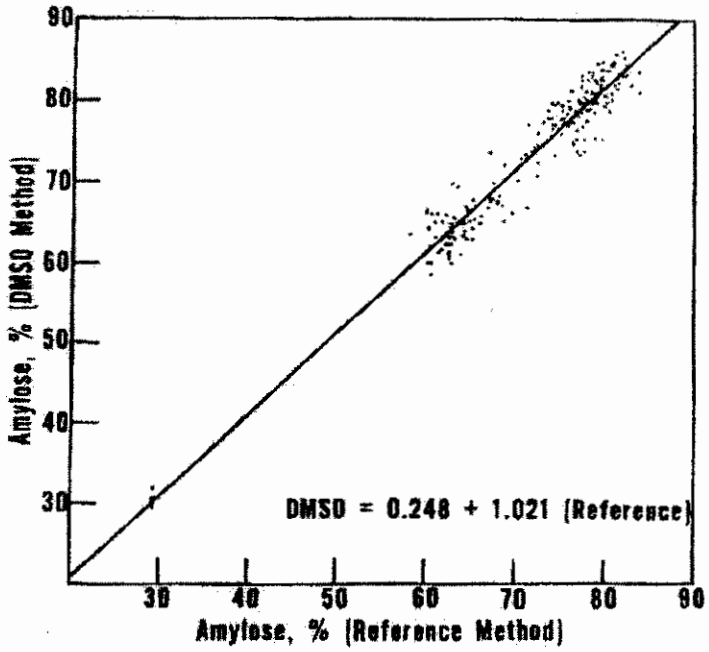


Fig. 3. Percent amylose in normal corn and amylomaize. Curve showing agreement in amylose determination between dimethyl sulfoxide (DMSO) method and a reference method.

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Comparison of Methods of Amylose Analysis

Amylose analyses by the DMSO method are compared in Fig. 3 with analyses run on identical samples by a method similar to that of Williams et al. (18), who used it to determine amylose in rice.

The slope of the curve (Fig. 3) is 1.02, indicating good agreement between the two methods. As the slope shows, amylose values by the DMSO method are higher than those by the reference method. The absorbance of a given starch sample is the same regardless of whether the starch was originally dissolved in dilute alkali or in 90% DMSO. Consequently, high results with the DMSO procedure are due to more accurate determination of starch by direct polarimetric analysis of DMSO extracts than by the indirect gravimetric procedure used in the reference method.

Microscopic Examination of Ground Corn Residues

Effectiveness of starch extraction was checked by inspection of ground tissue residues. After 3 to 6 min. of grinding in DMSO, endosperm cells were completely broken down; starch granules were swollen or dispersed; no birefringent starch granules remained. Residual cell fragments with swollen starch trapped in the protein network were negligible in amylomaize residues; but in ordinary corn and in waxy corn, some swollen cell fragments originating from horny endosperm cells were found.

Endosperm cell walls are readily broken by grinding in DMSO and do not interfere with starch extraction. Some of the swollen starch, originating in the horny endosperm, is still held mechanically in the protein network of cell wall-free cell contents. Because DMSO readily dissolves the zein bodies, small voids are left in the protein network (19); the fibrous matrix protein does not appear to be disrupted by the solvent. McGuire and Erlander (11) considered it necessary to digest endosperm protein with Pronase to obtain quantitative starch extraction. However, residual starch may be freed from the protein network by thorough grinding or long shaking in DMSO without previous protein digestion. The protein network survives prolonged treatment.

After the ground corn was shaken in DMSO for 24 hr., residues of amylomaize were generally microscopically free of starch. Ordinary corn and waxy corn still showed small amounts of highly swollen undispersed starch material adhering to fragments of protein network.

In general, the rate of solubilization of starches by grinding different varieties of corn in DMSO was as follows: amylomaize> waxy maize>ordinary corn.

Effect of Precipitation

Starches ordinarily require thorough extraction with a polar solvent, such as methanol, to extract most of the fatty acids which interfere with iodine sorption. In our method, this time-consuming extraction is replaced by a rapid, simple procedure involving precipitation of starch from DMSO extracts. Fatty acids remained in solution and were discarded with the supernatnat liquid.

Data are summarized in Table I, showing effectiveness of this procedure as compared with conventional solvent extraction of ground corn as a means of extracting fatty acids. Amylose values of precipitated DMSO starches were equivalent to those obtained by analysis of solvent-extracted corn.

TABLE I. EFFECT OF STARCH PRECIPITATION vs. SOLVENT EXTRACTION ON AMYLOSE DETERMINATION IN WHOLE CORN AND ENDOSPERM

Variety	Amylose, Not Defatted, Precipitated %	Amylose, Not Defatted, Not Precipitated %	Amylose, Defatted[a], Precipitated %	Amylose, Defatted[a], Not Precipitated %	Part Analyzed
Amicorn 6	60	49	61	59	Whole kernel
Amicorn 8	78	69	81	78	
Dent corn	27	23	28	27	
Amicorn 6	61	55	60	59	Endosperm
Amicorn 8	80	68	79	77	
Dent corn	27	24	28	27	
Average	55.5	48.0	56.2	54.5	

[a]Extracted with hot 85% methanol.

Analysis of Separate Parts of Kernel

Dissection of kernels to isolate endosperm for analysis not only is time-consuming, but also results in loss of some starch. However, grinding whole corn in DMSO, to avoid the work of dissection, may release optically active substances in addition to starch, excessive amounts of lipid, or other materials that might interfere with either measurement of the optical rotation or absorbance. Polysaccharides, sugars, proteins, and lipids concentrated in the germ or pericarp are possible sources of interference.

To test the over-all contribution of DMSO-extractable substances of germ and pericarp in amylose analysis, numerous assays were made of whole corn in comparison with isolated endosperm. A typical experiment with corn at three levels of amylose is shown in Table I. There was no difference in percent amylose when whole kernels or endosperm alone were assayed. Therefore, neither the germ, despite its high content of lipids and soluble sugars, nor the pericarp with its high pentosan content interferes with amylose determination in endosperm starch.

Noninterference of germ and pericarp with determination of amylose in endosperm starch was confirmed by analysis of DMSO extracts of hand-isolated germ and pericarp tissues. Pericarp extracts showed no optical activity, indicating that hemicelluloses, which make up about 50% of the pericarp, are not extractable with DMSO.

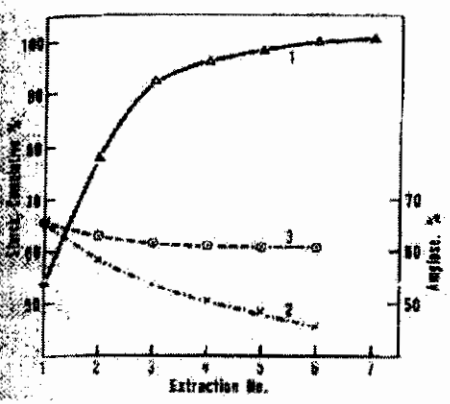
Fractional Extraction

Leach and Schoch (9) showed with pure starches that the linear fraction dissolved faster in DMSO than did the branched starch polymer. Similarly, we found in the fractional extraction of either amylomaize or ordinary corn that the first starch extracted was more linear than subsequent fractions (Figs. 4 and 5, curve 2). Because of this fractionation of starch during extraction, it was necessary to establish how much of the total starch (Figs. 4 and 5, curve 1) must be extracted to show an amylose content typical of that for the corn.

In amylomaize, amylose content of successive fractions extracted with DMSO

)LVENT
LE CORN

ot itated	Part Analyzed
	Whole kernel
	Endosperm
.5	



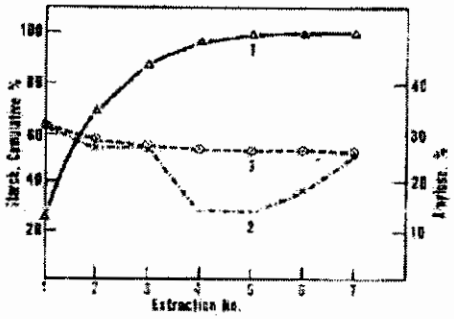


Fig. 4 (left). Fractional extraction of amylomaize. Triangles indicate starch, cumulative percent; X, percent amylose for respective extraction number; circle with dot, percent amylose, weighted average calculated from data for respective extraction plus previous extractions.

Fig. 5 (right). Fractional extraction of ordinary maize. Legend same as for Fig. 4.

varied from 65 to 46% (Fig. 4, curve 2). A weighted average of 61% amylose (Fig. 4, curve 3) was calculated from the entire series of individual fractions recovered in the experiment. This is in good agreement with an amylose content of 62% as determined by our procedure.

In ordinary corn, the pattern of starch extraction differs from that in amylomaize (Fig. 5). Amylose content of most of the starch extracted is between 25 and 30% (Fig. 5, curve 2). However, most of the remaining starch has an amylose content of about 15% (Fig. 5, curve 2). The pattern of extraction shown in the curves of Fig. 5 is in agreement with microscopic observations of DMSO-extracted residues of ordinary corn. Unextracted starchy material stains reddish, which is typical of amylopectin.

The data show that quantitative recovery of starch from maize is not necessary. Amylose values typical of the corn sample can be obtained with about 70% starch recovery in ordinary corn and 90% in amylomaize. Actual starch recoveries in our method are in excess of 90 and 95%, respectively, for ordinary corn and amylomaize with only a 3-min. grinding period. By extension of the grinding period, quantitative starch recoveries are approached.

Acknowledgments

We are indebted to J. R. McCarthy for help in determining the specific rotation of starch and to Alvena Stoutenborough and Florence McClellan for performing many of the analyses.

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EXHIBIT 13
A204 – A207

A Simplified Colorimetric Procedure for Determination of Amylose in Maize Starches

C. A. KNUTSON[1]

ABSTRACT

When iodine is dissolved in mixtures of water and dimethylsulfoxide (Me_2SO), triiodide ion is formed; the extent of triiodide formation is dependent upon the proportion of Me_2SO in the mixture. A method has been developed to utilize this reaction to form the blue amylose-iodine complex, providing a simple procedure for quantitative measurement of amylose in starches. Starch is dissolved in a mixture of 90% Me_2SO and 10% water containing iodine. Upon dissolution of the starch, the mixture is diluted with water to give a final Me_2SO concentration of 10%, whereupon the blue amylose-iodine complex forms immediately. Amylose is then determined by measurement of the absorbance of the complex at 600 nm. Use of Me_2SO to generate triiodide ion eliminates the necessity for preparing buffered solutions of iodine in potassium iodide. Accuracy and sensitivity of the procedure is equivalent to that of the conventional methods of preparing the amylose-iodine complex.

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Determination of the amylose content of starches is commonly accomplished by measurement of the blue amylose-iodine complex. This measurement can be done quite precisely by potentiometric titration (Banks et al 1971, Knutson et al 1982) but is more conveniently done colorimetrically. The first colorimetric procedure for quantitative estimation of amylose was reported by McCready and Hassid (1943). Because of the diversity of starches from different sources, many modifications of the original procedure have been reported. Commonly used procedures for analysis of cereal starches are described by Williams et al (1970) and Wolf et al (1970). A recent report by Morrison and Laignelet (1983) describes an improved method that is rapid and accurate, and which provides a means for determination of both apparent amylose (measured in the presence of lipids) and total amylose (measured on lipid-free starch).

Formation of the amylose-iodine complex requires the presence of triiodide (I_3^-) ions in the reaction mixture to initiate the reaction (the actual nature of the iodine species in the complex has been extensively studied but not yet definitively determined). In all of the colorimetric procedures currently in use, triiodide is formed by dissolving iodine and potassium iodide in buffered solutions at neutral or slightly basic pH. However, it is possible to form the triiodide ion in a variety of basic solvents (e.g., pyridine) without addition of potassium iodide. The basic solvent, B, causes partial solvolysis and disproportionation of the I_2 molecule to give I^- and BI^+, whereupon the I^- reacts with the remaining I_2 to form the triiodide ion.

Dimethylsulfoxide Me_2SO, which is a moderately strong Lewis base, is known to form triiodide ion (Klaeboe 1964, Courtot-Coupez and Madec 1971) and is also an excellent solvent for starches (Wolf et al 1970). Thus, if both starch and iodine are dissolved in Me_2SO, an opportunity is presented to form the amylose-iodine complex in a single step, without the necessity of preparing buffer solutions. In addition, by avoiding the use of buffers, the risk of aggregation of the amylose-iodine complex is reduced. This paper describes the stoichiometry of the formation of triiodide ion in Me_2SO-water mixtures and the conditions necessary for formation of the amylose-iodine complex in such mixtures, and presents a rapid single-step method for determination of the amylose content of starches of widely varying composition. Some speculation regarding the nature of the iodine species involved in the complex formation is also included.

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MATERIALS AND METHODS

Iodine, potassium iodide, and Me_2SO were reagent-grade chemicals from commercial sources, used without further purification. The standard amylose sample had been extracted at 70° C from defatted corn starch and crystallized from an aqueous solution saturated with 1-butanol; molecular weight (by intrinsic viscosity) was 1.76×10^5, equivalent to a degree of polymerization (DP) of 1,100. Samples of dent corn, waxy maize, amylomaize V (50% amylose), and amylomaize VII (70% amylose) starches were obtained from American Maize-Products Co., Hammond, IN. Defatted starches were prepared by dissolving starch in 90% Me_2SO, precipitating with absolute ethanol, and redissolving in 90% Me_2SO.

Spectrophotometric measurements were done with a Cary model 14 recording spectrophotometer in 0.5-cm cells. Routine colorimetric measurements were done with a Beckman model DU spectrophotometer in 1-cm cells or with a Beckman model B spectrophotometer equipped to hold 19-mm matched test tubes.

Determination of total carbohydrate content of native and defatted samples in solution was done by the phenol-sulfuric acid method of Dubois et al (1956).

The stoichiometry of triiodide ion formation in Me_2SO-water mixtures was evaluated by measuring absorbance spectra of iodine solutions, from 0 to $6 \times 10^{-4} M$, in 0–100% Me_2SO, both in the presence and absence of potassium iodide (KI). The molar absorptivity of I_3^- in Me_2SO-water mixtures of varying composition was determined by extraction of a $1.25 \times 10^{-3} M$ iodine solution in hexane with an equal volume of Me_2SO-water mixture containing 0.01 M KI. Hexane is almost totally immiscible with Me_2SO-water mixtures. Any I_3^- formed was extracted into the Me_2SO-water layer, and unreacted I_2 remained in the hexane layer. The layers were separated and absorbance of each measured immediately. The amount of unreacted I_2 was determined by measuring the absorbance at 522 nm (molar absorptivity 900 in hexane). Absorbance of I_3^- in the Me_2SO-water layer was measured at maximum, 350–365 nm, and the molar absorptivity was calculated from the known amount of iodine that had been lost from the hexane layer. The same extraction procedure, using Me_2SO-water mixtures containing no KI, was used to evaluate the equilibrium of the I_3^- formation reaction.

The amylose-iodine complex was formed by dissolving 400 μg amylose in 10 ml of Me_2SO-water mixtures that contained varying iodine concentrations. The absorbance spectra were measured, and optimum conditions for complex formation were identified. These conditions were then incorporated into the following standard procedure, which was used to analyze starch samples of varying amylose content. Starch sample (1–5 mg) is dissolved in 10 ml 90% Me_2SO containing $6 \times 10^{-3} M$ iodine. Purified starch will dissolve overnight at room temperature; ground whole corn or endosperm requires heating to 50° C overnight. One milliliter of this solution is diluted with 8 ml of water and mixed thoroughly. The complex forms immediately and stabilizes to maximum absorbance within

30 min. After 30 min, absorbance is measured at 600 nm. At this wavelength, absorbance is 99% of maximum (at 620 nm), and it is not necessary to use such a wide slit width on the spectrophotometer. Apparent amylose, uncorrected for amylopectin, is calculated from a standard curve obtained with pure amylose; this value is corrected for amylopectin by the equation:

$$\% \text{ Amylose} = \frac{\% \text{ Apparent amylose} - 6.2}{93.8}.$$

RESULTS

Absorbance of the triiodide ion in varying concentrations of iodine and Me_2SO is shown in Figure 1. In 100% Me_2SO the absorbance changes linearly with iodine concentration. At lower Me_2SO content, there is an initial lag in I_3^- formation until iodine concentration reaches $2 \times 10^{-4} M$, beyond which absorbance again varies linearly with iodine concentration, with the extent of variation dependent upon the Me_2SO content of the solution. Maximum absorbance in 100% Me_2SO occurs at 365 nm and shifts downward as Me_2SO content is decreased, to 350 nm at the lowest detectable absorbance.

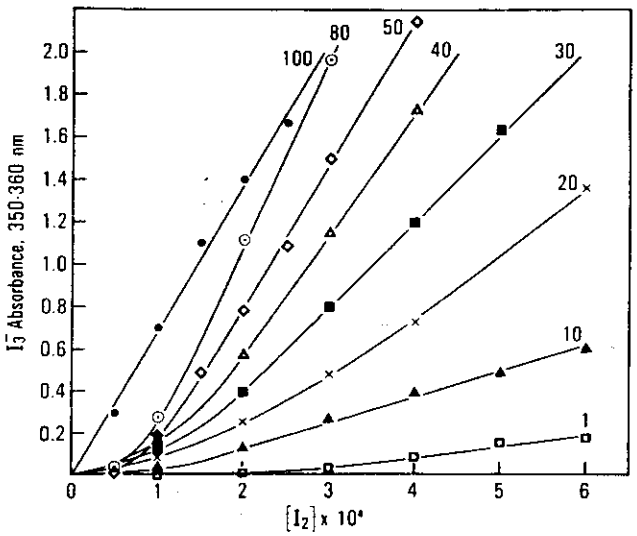

Fig. 1. Variation of absorbance with iodine concentration in Me_2SO-water mixtures: ●—●, 100% Me_2SO; ⊙—⊙, 80%; ◇—◇, 50%; △—△, 40%; □—□, 30%; x—x, 20%; ▲—▲, 10%; □—□, 1%.

TABLE I
Effect of Potassium Iodide on the I_3^- Absorbance in Me_2SO-Water Mixtures[a]

		KI Concentration, Molar			
% Me_2SO	λ max	0	10^{-3}	10^{-2}	10^{-1}
0	353	0	0.540	0.942	1.070
10	355	0.054	···	0.845	1.005
50	360	0.248	0.680	0.885	0.998
100	365	0.702	1.068	1.126	1.226

[a] Initial iodine concentration $5.1 \times 10^{-5} M$; path length 1 cm.

TABLE II
Molar Absorptivity of I_3^- in Varied Concentrations of Me_2SO-Water

% Me_2SO	Molar Absorptivity
0	22,306
10	23,172
20	26,417
30	24,919
40	26,493
50	26,118
60	25,663
80	26,811
100	26,411
Mean (20–100%)	26,119
SD	637.8

The addition of KI to iodine-Me_2SO-water mixtures increases the absorbance, as shown in Table I. When I^- is provided in the form of KI, more I_2 can react directly to form I_3^-; at I^- concentrations higher than $10^{-2} M$, essentially all I_2 present can go into I_3^- formation. That the absorbance is not constant at the higher KI concentrations indicates a change in the equilibrium of the reaction with varying Me_2SO content, or a variation in the molar absorptivity of I_3^-.

To determine the molar absorptivity of I_3^- at varying concentrations of Me_2SO, the hexane extraction procedure described in the Methods section was used. Results are given in Table II. The molar absorptivity of I_3^- in aqueous solutions is 26,400 (Awtrey and Connick 1951). The values in the table agree well with this value, except at the lowest concentrations of Me_2SO. However, under the conditions of this experiment, little I_3^- is formed and extracted into the Me_2SO-water layer at these Me_2SO concentrations (0 and 10%), so the absorbance measurement is subject to correspondingly larger errors. If the 0 and 10% Me_2SO values are disregarded, the average of the remaining values is 26,119, which is in good agreement with the reported value.

The same extraction experiment without KI in the mixtures was then conducted to study the equilibrium of the I_2-I_3^- formation at varying concentrations of Me_2SO. Theoretical maximum I_3^- concentration was calculated assuming two I_2 molecules consumed for every I_3^- ion formed. Results are shown in Table III. Above 80% Me_2SO, I_3^- formation is essentially 100%, whereas below 10% Me_2SO there is essentially no I_3^- formed. In the intermediate concentrations I_3^- variability is dependent upon Me_2SO concentration, and there is a significant amount of iodine not accounted for either as I_2 or I_3^-. This iodine presumably has been disproportionated to form I^- but has not reacted with molecular iodine to form I_3^-.

Various compositions of I_2-Me_2SO-water were then evaluated for their capacity to form the amylose-iodine complex. Absorbance values for solutions of 400 μg amylose in 10 ml of Me_2SO-water mixtures containing 1 to $6 \times 10^{-4} M$ iodine are shown in Figure 2. Amylose-iodine complex could be formed in Me_2SO concentrations up to 40%. Maximum binding occurred with Me_2SO concentrations of 10% or less and at iodine concentrations of 4×10^{-4} or higher. In 40% Me_2SO maximum absorbance was at 570 nm, and at 10% or less the maximum was at 620 nm. In 40% concentrations the complex formed very slowly, requiring 60–90 min to reach maximum absorbance.

A sample of waxy maize starch, previously identified as amylose-free, was analyzed under the conditions of optimum amylose-iodine complex formation, i.e., $6 \times 10^{-4} M$ iodine in 10% Me_2SO, to determine the extent of interference from amylopectin. The

TABLE III
Concentration of I_2 and I_3^- at Equilibrium in Varied Concentrations of Me_2SO-Water[a]

	Concentration, $M \times 10^3$			
% Me_2SO	$[I_2]$	$[I_3^-]$	Total $[I_2] + 2 \times [I_3^-]$	% of Theory
0	1.26	0	1.26	100.9
10	1.18	0.003	1.19	95.0
20	1.12	0.006	1.13	90.6
30	1.05	0.015	1.08	86.5
40	0.888	0.044	0.976	78.2
50	0.531	0.171	0.872	69.9
60	0.347	0.287	0.921	73.8
70	0.154	0.500	1.15	92.5
80	0	0.617	1.23	99.0
100	0	0.617	1.23	99.0

[a] Original iodine concentration, $1.25 \times 10^{-3} M$.

absorbances of amylose and amylopectin under these conditions are shown in Figure 3. Amylopectin has a small absorption band with a maximum at 520 nm and an extinction coefficient at 600 nm that is 6.2% that of amylose. On the basis of this evaluation, the standard procedure was established for the routine analysis of starch samples. The standard procedure was used to analyze both native and defatted samples of maize starches of varying amylose content. These results were compared to values obtained using conventional I_2-KI reagents as described by Wolf et al (1971) and Knutson et al (1982). Comparative results are shown in Table IV. The values obtained with iodine-Me_2SO on defatted starches are equivalent to the other procedures; native samples of high-amylose starches do not agree as well and have significantly higher standard deviations.

DISCUSSION

Stoichiometry of I_3^- Formation

The formation of I_3^- in Me_2SO solutions has been recognized for some time. Klaeboe (1964) mistakenly assumed that it was caused by impurities in the Me_2SO. Courtot-Coupez and Madec (1971) evaluated the formation of triiodide in Me_2SO-water mixtures in the presence of 0.1 *M* lithium perchlorate and speculated on the nature of the ionic species involved. The reaction is most probably analogous to the disproportionation of Cl_2 in Me_2SO described by Gavreau et al (1980). Such a reaction would proceed thusly:

$$\text{A)}\ (CH_3)_2SO + I_2 \rightleftarrows (CH_3)_2SO\text{-}I^+ + I^-.$$

The iodide thus formed would then combine with available molecular iodine in the usual manner for triiodide formation:

$$\text{B)}\ I^- + I_2 \rightleftarrows I_3^-$$

and the overall reaction would be

$$\text{C)}\ (CH_3)_2SO + 2I_2 \rightleftarrows (CH_3)_2SO\text{-}I^+ + I_3^-.$$

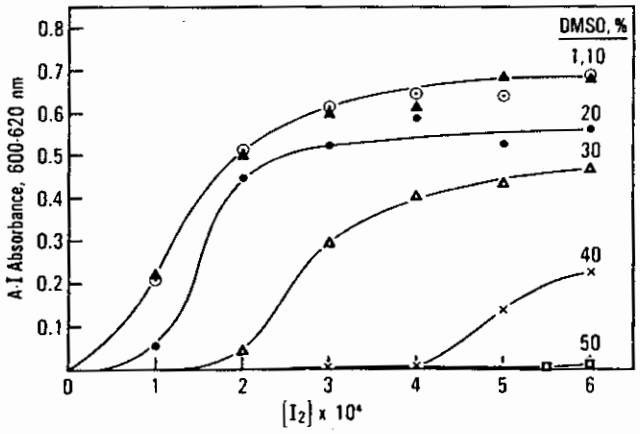

Fig. 2. Absorbance of amylose-iodine complex in varying concentrations of iodine and Me_2SO: ⊙——⊙, 1% Me_2SO; ▲——▲, 10%; ●——●, 20%; △——△, 30%; x——x, 40%; □——□, 50%.

The fact that the formation of I_3^- must occur in two steps serves to explain the lag in I_3^- formation observed in the data in Figure 1 and Table III. There must be an increase in either Me_2SO or I_2 to drive reaction A toward I^- formation; only after sufficient I^- is available, as well as sufficient unreacted I_2, can reaction B proceed to form I_3^-. As shown in Table III, all the I_2 in the system remains unreacted in the absence of Me_2SO, and is all converted to I_3^- in Me_2SO concentrations of 80% or higher.

It must be noted that the equilibrium established by extraction from hexane is quite different from that established in Me_2SO-

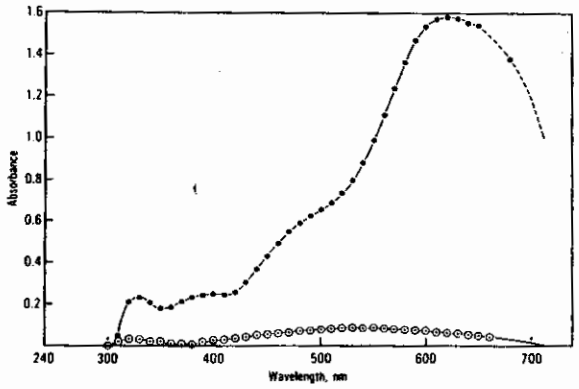

Fig. 3. Absorbance spectra of amylose-iodine complex (●——●) and amylopectin-iodine (⊙——⊙). Carbohydrate 40 mg/ml, 10% Me_2SO, 6 $\times 10^{-4}$ *M* I_2.

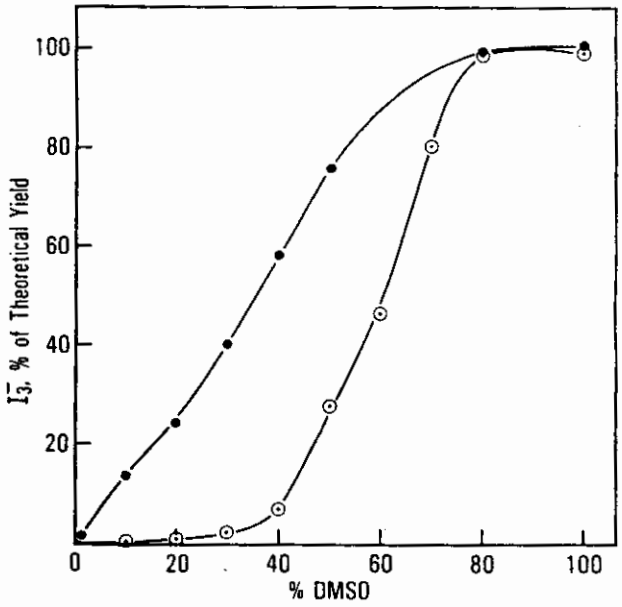

Fig. 4. Formation of I_3^- with increasing Me_2SO concentration: ●——●, single phase reaction; ⊙——⊙, hexane extraction reaction.

TABLE IV
Comparison of Amylose Values Obtained by Measurement of Amylose-Iodine Complex Formed in I_2-Me_2SO and in I_2-KI

	Amylose Content, %											
	I_2-Me_2SO						I_2-KI (defatted samples only)					
	Native			Defatted			Method A[a]			Method B[b]		
Sample	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD	*n*	Mean	SD
Dent starch	8	23.6	1.7	5	22.9	1.2	10	21.9	1.5	8	23.0	0.9
Amylomaize V	8	47.9	4.5	5	48.9	1.3	8	48.8	2.1	5	51.4	2.0
Amylomaize VII	8	66.3	7.1	5	70.1	2.0	8	65.1	2.7	5	68.2	3.0

[a] Method A, Wolf et al, 1970.
[b] Method B, Knutson et al, 1982.

water mixtures alone at low Me_2SO concentrations. This is illustrated in Figure 4, where I_3^- concentrations from Figure 1 (at $3 \times 10^{-4} M$) and Table III are expressed as percentages of the theoretical value and plotted versus Me_2SO concentration. This difference in equilibria is to be expected in view of the low solubility of molecular iodine in water; at low Me_2SO concentrations, I_2 is retained in the hexane layer, and the equilibria of reactions A and B are shifted to the left.

Nature of the Amylose-Iodine Complex Formed in Me_2SO-Water Mixtures

It must be assumed that when the amylose-iodine complex is formed in solution, the ratio of I_2 to I_3^- in the complex reflects the composition of the solution, because deviation from this composition is energetically unfavorable. We have previously relied on this assumption to estimate iodine species involved in aqueous I_2-KI systems from potentiometric and spectrophotometric data (Knutson et al 1982). In the present study, only spectrophotometric data are available. This precludes precise calculation of I_2/I_3^- ratios because of the large disparity between the absorptivity of I_3^- and I_2 (746 at 445 nm). Estimated values calculated from spectrophotometric data of Me_2SO-water mixtures between 10 and 50% Me_2SO ranged from an I_2/I_3^- ratio of 11 at 10% Me_2SO to 2 at 50%. Although these calculations may not be accurate, they do reflect the trend of the system, and the variation in absorbance of the amylose-iodine complex formed in different Me_2SO concentrations (Fig. 2) demonstrates the relative effectiveness of the different I_2/I_3^- compositions to form the amylose-iodine complex.

It is significant that in this and our previous work (Knutson et al 1982) there is a downward shift in the absorbance maximum of the amylose-iodine complex as the triiodide content of the solution is increased, indicating a change in the nature of the complex as more of the ionic species is incorporated.

Variability in Analysis of High-Amylose Starches

The data in Table IV show considerable variability between native and defatted high-amylose starches, although the difference is minimal in dent starch. This variability is undoubtedly caused by the interference of lipids, as high-amylose starches are known to contain more lipid than normal starches (Acker and Schmitz 1967, Tan and Morrison 1979, Morrison and Milligan 1982). Therefore, if accurate results are to be obtained, defatting of the sample is necessary with this method. The results on native samples do demonstrate the usefulness of the method without defatting for initial screening to assign samples to different amylomaize classes.

Applicability of the Method to Other Starches

This method was developed for analysis of maize starches and has only been tested on maize starches. However, it can be safely assumed that use of iodine-Me_2SO as a substitute for I_2-KI will be applicable to any starch, and that successful adaptation to other starches merely requires satisfactory procedures for dissolving and defatting the starch.

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A208 – A220

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CHANGES IN STARCH GRANULE SIZE AND AMYLOSE PERCENTAGE DURING KERNEL DEVELOPMENT IN SEVERAL *ZEA MAYS* L. GENOTYPES[1]

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ABSTRACT

Amylose concentration in starch reportedly increases with increasing physiological age of the tissue in which the starch is synthesized. Analyses of starches from developing maize (*Zea mays* L.) endosperms of two independently occurring *amylose-extender* (*ae*) alleles, named *ae-Ref* (Reference) and *ae-il* (induced-1), the double mutant *amylose-extender sugary (ae su)*, and *normal* were consistent with this developmental pattern; however, the mutant *waxy (wx)* and the double mutant *amylose-extender waxy (ae wx)* were exceptions. The apparent amylose percentage of *wx* remained near zero and that of *ae wx* decreased during kernel development. Maximum starch granule size from all genotypes increased during kernel development from 18 to 36 days post-pollination, but granule size distributions varied among genotypes. *Normal* and *wx* kernels produced the largest granules. Compared to *normal*, the maximum granule size was reduced in *ae-Ref*, *ae-il*, and *ae wx*, resulting in an increased frequency of medium to small granules. Granules from *ae su* kernels generally were very small, although a few granules were as large as those from *normal*. When starch granules from 36-day-old *ae-Ref*, *ae su*, and *normal* kernels were separated into different size classes, a decline in apparent amylose percentage with decreasing granule size was observed. Thus, the smaller granules reflected the characteristics of unfractionated starch isolated from whole endosperms earlier in kernel development, supporting the hypothesis that these small granules were derived from physiologically younger cells.

Endosperm cells of a developing maize (*Zea mays* L.) kernel are comprised of a population of cells of varying physiological ages (1,2,3). The basal endosperm cells begin starch biosynthesis late in kernel development and contain small starch granules (1). Peripheral endosperm cells, which are the last to develop, also contain small starch granules (2). Thus, two gradients of cellular development exist in maize kernels: a major gradient from the central crown part of the kernel to the point of attachment of the kernel, and a minor gradient from the central kernel outward to the peripheral endosperm cells. However, granules within a given cell from *normal* maize kernels are similar in size (3).

Since all endosperm cells are not the same age, the physiologically younger cells may undergo the same developmental changes in starch biosynthesis as older cells but at a later time in kernel development. Shannon (4) subdivided endosperm from 30-day-old *normal* kernels into seven zones from the point of kernel attachment to the crown. Endosperm zones from near the base contained high amounts of soluble sugar and low amounts of starch, similar to the carbohydrate composition reported for whole endosperms 8, 10, and 12 days

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post-pollination (5). The low sugar, high starch content of the upper endosperm zones was similar to the carbohydrate composition of whole endosperms 22 and 28 days post-pollination (5). Furthermore, incorporation of ^{14}C into starch granules per unit granule surface area was similar for all starch granules larger than those containing approximately 0.1 γ of starch per granule (3). Smaller granules contained only half as much ^{14}C per unit granule surface. From these data and cytological observations, it was suggested that the smallest granules may have been derived from physiologically younger cells which had not developed full starch biosynthetic capacity (3).

Another developmental change observed in various plant species has been an increased amylose concentration with increasing age of the tissue from which the starch was isolated. For example, Badenhuizen and Chandorkar (6) found that the amylose content of tobacco-leaf starches increased as older leaves were sampled. Similarly, wheat-kernel starch showed an increased amylose percentage from 2 to 6 weeks after anthesis (7), and Banks *et al.* (8) found a doubling in amylose percentage in barley kernel starch from 9 to 46 days after anthesis. Increasing amylose concentrations were similarly observed in both *normal* and amylomaize (hybrids with the *amylose-extender* gene) starches (6,9) isolated from kernels of advancing development. However, developmental changes in amylose content for other maize endosperm mutants which differ markedly in starch properties (10) have not been evaluated.

If physiologically younger cells lack full biosynthetic capacity as suggested previously (3), amylose concentration in starch from older endosperms would be predicted to vary as a function of granule size. To test this hypothesis and evaluate changes in amylose percentage during development of additional maize mutants, the patterns of amylose accumulation were investigated for six maize genotypes, and the amylose content was determined in different starch granule size classes separated from endosperms collected 36 days post-pollination.

MATERIALS AND METHODS

Genetic Material

The dent maize inbred W64A (normal); two *amylose-extender* alleles, named *ae-Ref* and *ae-il*, which have different amylose contents at maturity (11); *waxy (wx)*; and the double mutants *amylose-extender sugary (ae su)* and *amylose-extender waxy (ae wx)* were used. These genotypes had been backcrossed to W64A 9, 6, 8, 7, and 4 times, respectively. The *ae su* and *ae wx* double mutants contained the *ae-Ref* allele. *Normal* contained the dominant alleles at these gene loci and thus had the genotype *Ae Ae Su Su Wx Wx*. The plant materials were grown in 1972 at The Pennsylvania State University Rock Springs Agricultural Research Center in a split-plot design. The post-pollination dates of harvest were used as the main plots, and genotypes were used as the subplots. Two ears per genotype were sampled from each of two replications by date. All plants were self-pollinated. Ears were harvested at 18, 24, and 36 days after pollination and frozen in ethanol cooled with Dry Ice within 5 min after removal from the plants. Subsequent storage was at −25° C. Entire ears were freeze-dried and samples of 50, 30, and 20 kernels were removed from the central portion of ears 18, 24, and 36 days post-pollination, respectively.

Endosperms free of pericarp and embryo tissues were soaked overnight in 60

ml of "steep" solution (0.01*M* $HgCl_2$ and 0.02*M* sodium acetate, pH 6.5) in a 40°C water bath. The softened endosperm tissue was ground by means of a porcelain mortar and pestle. The slurry was filtered through Nitex (Tobler, Ernst and Traber, Inc., New York, N.Y.) nylon bolting cloth with sieve openings of 80 μ, and released starch granules were washed through the screen with water. The fibrous material not passing through the screen was returned to the mortar, and the grinding and filtration processes were repeated until essentially all granules were removed from the fibrous material.

Starch granules passing through the 80-μ mesh screen were suspended in a 0.05*M* NaCl solution and purified by shaking repeatedly for 1 hr each time with toluene (12). After centrifugation (1000 × *g*, 15 min) most of the starch was pelleted, while denatured protein plus some starch was located at the interface of the toluene and saline solutions. The material located in the first two interfaces was collected and combined. The trapped starch granules were released by gentle shaking for 1 hr, followed by centrifugation (1000 × *g*, 10 min). Recovered granules were combined with the pelleted granules. Since the bulk of the protein was removed with the first two toluene extractions, very little starch was trapped in the subsequent toluene washes. Thus, no further attempts were made to recover the few granules which may have been at the interface. Seven toluene extractions for 18-day-old kernels and ten extractions for 24- and 36-day-old kernels gave a clear toluene-saline interface by the final shaking. After the final shaking, the purified starch granules were washed four times with water by suspension and centrifugation as described above, and stored under toluene at 4°C. Starch preparations were checked for purity and freedom from starch granule clumping by light microscopy.

Starch Granule Size Distributions

Size distributions were generated on a Model B Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) which was standardized by using synthetic latex beads with a reported diameter of 3.49 μ, and by using paper mulberry pollen (*Broussonetia papyrifera* L., Vent.) with a reported diameter of 12 to 13 μ. The number of granules having diameters from 1.5 to 2.5 μ, from 2.5 to 3.5 μ, etc., were determined, and these values used to calculate the points plotted at 2 μ, 3 μ, etc., respectively, on the granule size distribution figures. Points were generated every 1 μ from 2 μ upward until a maximum size was determined. A size distribution was determined on a single sample from the two ears in the first replication for each genotype at each age. Granule sizes are presented as percentages of the total number of granules counted. Over 50,000 granules were counted for each distribution (Table I).

Separation of Starch Granules by Size

Starch samples from kernels 36 days post-pollination from a single ear of the genotypes *ae-Ref, ae su, ae wx*, and *normal* were washed with acetone, dried, and gently powdered with a mortar and pestle. Starch granules were separated by sifting through sieves with pores of 20, 10, and 5 μ into sizes ranging from 20 to 10 μ, 10 to 5 μ, and smaller than 5 μ. An ATM Allen Bradley Sonic Sifter (Fisher Scientific, Pittsburgh, Pa.) was used. Approximately 100 mg of *ae-Ref* and *ae su* granules passing through the 5-μ sieve were further separated by settling through an aqueous glycerol mixture. These granules were suspended in 20 ml of distilled

water, carefully layered onto 30 ml of 30% glycerol in a 3 × 20-cm glass test tube, covered, and placed in the cold (0°–5° C) overnight. The next day the upper 30 ml was collected as fraction C-1 and the middle 10 ml as C-2. The remaining 10 ml was centrifuged, the supernatant discarded, the starch pellet suspended in 20 ml of distilled water, and the settling procedure repeated. The upper 30 ml of this settling was combined with C-1, the middle 10 ml with C-2, and the bottom 10 ml was collected as C-3.

Determination of Amylose Percentage

Apparent amylose percentages of dimethyl sulfoxide dispersed starch samples were determined by the blue value method of Wolf *et al.* (13) except that the quantity of KIO_3 was increased as previously described (11), and starch concentrations were determined by the phenol sulfuric acid test (14). Duplicate kernel samples from each ear were analyzed. All amylose percentage determinations were done in duplicate.

Statistical Analysis

Analyses of variance were performed using standard techniques (15). Waller

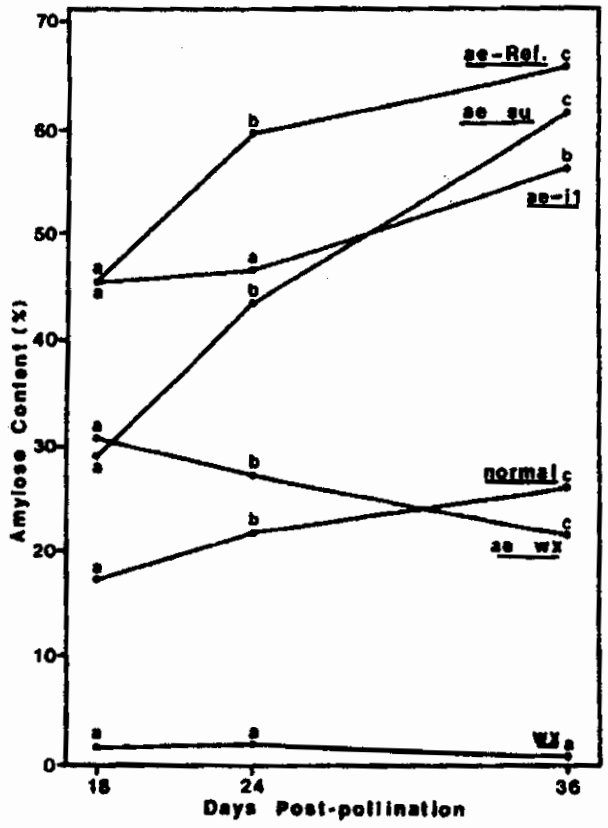

Fig. 1. Changes in amylose content (%) in endosperm starches of six maize genotypes during development. Points on the same line marked by the same letter are not significantly different (k = 100).

and Duncan's Modified (Bayesian) Least Significant Difference Test (16) with $k = 100$ was used to determine mean separations.

RESULTS AND DISCUSSION

The apparent amylose percentages in starch from *normal, ae-Ref, ae-il,* and *ae-su* increased with increasing kernel age (Fig. 1) similar to the amylose changes in *normal* and *ae* kernels reported earlier (6,9). However, that from *ae wx* decreased. Starch from *wx* contained little amylose (approximately 1%) and did not change with increasing age. The rate of change in apparent amylose percentage with age varied with the genotype, and the double mutant *ae su* produced the greatest change (Fig. 1).

The two *ae* alleles had the same amylose percentage at 18 days, but differed after that time (Fig. 1). However, the rate of change for the two alleles from 24 to 36 days post-pollination was very similar, indicating that the difference noted in the mature starches (11) probably occurred between 18 and 24 days after pollination. Comparison of the amylose percentages of *ae-Ref* and *ae su* indicated that the *su* allele in combination with *ae* inhibited the *ae*-enhanced accumulation during early kernel development (up to 18 days), but that after that time the *ae* allele overcame the *su* effect and the percentage of amylose increased at a rate greater than that observed in the single mutant *ae-Ref*.

Starch of *ae wx* showed an unusual pattern of amylose change with increasing age (Fig. 1). As noted above, the *wx* allele effectively blocked amylose accumulation at all sampling times. However, the addition of the *ae* allele to *wx* produced starch with an apparent amylose of 31% at 18 days post-pollination. In contrast to the typical increase in amylose percentage during kernel development (6,9), *ae wx* amylose percentage declined (Fig. 1). Thus, in *normal, ae,* and *ae su* the rate of amylose accumulation was greater than that of amylopectin with increasing kernel age, but the reverse was true for *ae wx*.

Consideration of the changes in relative activity of various enzymes during endosperm cellular development is useful in interpreting these patterns of amylose accumulation (Fig. 1). The developing maize kernel is composed of cells of varying physiological ages (1,2,3) and cells in the earliest stages of starch biosynthesis may produce starch from UDP-glucose by the action of granule-bound UDP-glucose starch glucosyltransferase (3,5). Later in development the activity of soluble ADP-glucose-starch glucosyltransferase and phosphorylase II and III increases (5,17). Schiefer *et al.* (18) suggested that amylose is produced *in vivo* by the action of a soluble ADP-glucose-starch glucosyltransferase while amylopectin is produced by an enzyme complex of ADP-glucose-starch glycosyltransferase and branching enzyme. Also, based on a polyacrylamide gel electrophoresis study, they (18) showed that *ae* kernels contained higher activity in the free glucosyltransferase bands than did *normal*. Another theory for *ae* action given by Shannon and Creech (19) suggests that *ae* may contain a debranching enzyme which would remove branches from amylopectin yielding a higher proportion of amylose and low-molecular-weight linear polysaccharides. Regardless of the specific enzymes involved, the amylose percentage in the starch is the result of the relative activity of two or more enzymes or enzyme complexes. Thus, during cellular development it is likely that a change occurs in the relative activity of these enzymes resulting in the increased percentage of amylose in *ae-*

Ref, ae-il, ae su, and *normal*, and the decline in the percentage of apparent amylose in *ae wx* (Fig. 1).

Although others (10,20) have reported a near normal apparent amylose content in *ae wx* starch, Tsai (21) recently reported that unpublished data from his laboratory indicated that *ae wx* starch was exclusively amylopectin, but the maximum absorption of the starch-iodine complex was 580 nm as compared to 540 nm for *wx* starch. An amylopectin with an absorption maximum at 580 nm could have contributed to the apparent amylose estimated by the blue value procedure (13) used in the present study. If *ae wx* starch is composed only of amylopectin, then with increasing age either the average chain lengths decreased or the polysaccharides produced by physiologically older cells are more tightly branched. Additional study will be necessary to determine if *ae wx* starch contains only a modified amylopectin or a mixture of amylose and amylopectin.

The average granule sizes for *normal* and the various mutant starches (as determined by the Coulter Counter) varied from a minimum of 2.6 μ in diameter for *ae su* at 18 days post-pollination to 7.7 μ for *normal* at 36 days post-pollination (Table I). The average granule sizes were smaller than those reported by Wolf *et al.* (22) for *normal* and *ae* starches from mature kernels. This is not surprising since their samples were from mature kernels isolated by a commercial procedure which did not quantitatively recover the small granules. Mercier *et al.* (9) showed an increase in granule size with increasing kernel age. The average granule sizes of their *normal* and *ae* starch preparations were also larger than ours. This was probably because the starches for their study (9) were taken from the floury portion of kernels which contain the more mature cells (3). Brown *et al.* (23) determined the minimum and maximum granule dimensions of starches isolated from kernels 12, 18, and 24 days post-pollination. Average starch granule sizes, based on the measurement of 120 granules, were also larger than those we obtained with the Coulter Counter. These differences may have been due to the small sample size used by Brown *et al.* (23). Alternatively, our samples could possibly contain small nonstarch cellular constituents not observed when the starch preparations were microscopically checked for purity. Such an inclusion would inflate the number of small (1.5 to 2.5 μ) granules and result in a smaller overall average diameter. Although the average granule diameter given in this study may be smaller than previously reported (9,23), the great heterogeneity of granule size emphasizes the importance of changes in granule size distribution with increasing age.

The starch granule size distributions for *normal* and the various mutant types at 18, 24, and 36 days post-pollination are shown in Fig. 2. Actual numbers of granules counted to generate the distribution are given in Table I. Size distributions of *normal* starch granules at 18, 24, and 36 days after pollination indicated that the greatest change in granule size occurred between 18 and 24 days (Fig. 2a). However, at all times after pollination, significant numbers of the granules were less than 5 μ in diameter. Size distributions of starch granules from all mutant genotypes showed a general reduction in maximum granule size, and a repression of granule enlargement compared to *normal*, but those from *wx* most closely resembled *normal* (Fig. 2b through 2f). The *ae* genotypes produced smaller starch granules but the maximum granule size was larger in *ae-il* than in *ae-Ref* starch (Fig. 2c and 2d). Size distributions from *ae wx* (Fig. 2e) showed greater reduction in granule dimensions than either *ae-Ref* or *ae-il*. Furthermore,

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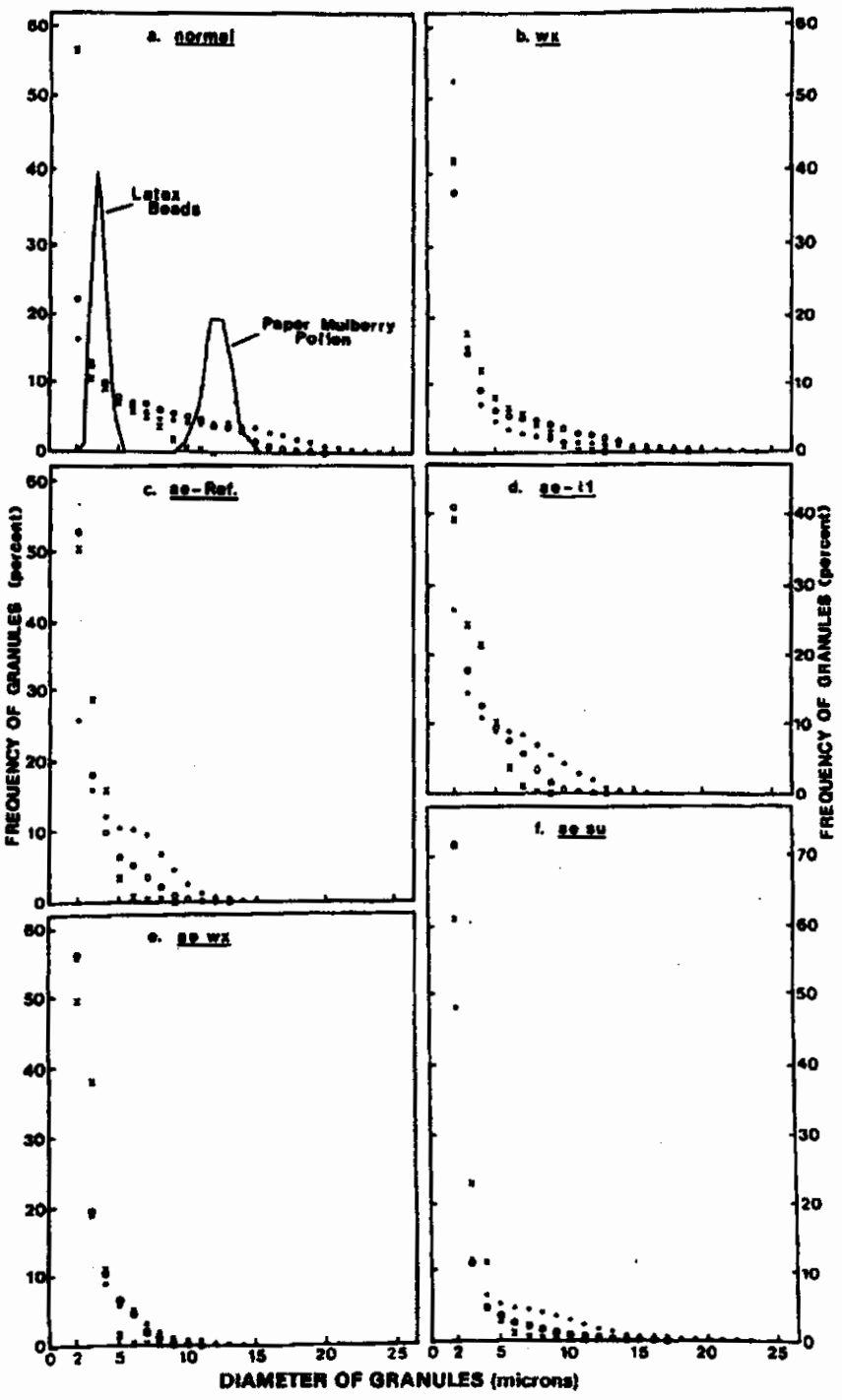

Fig. 2. Size distribution of starch granules of six maize genotypes at 18 (x's), 24 (open circles), and 36 (solid circles) days post-pollination. Solid lines in Fig. 2a represent size distributions for latex bead and paper mulberry pollen standards used for calibration.

very little granule enlargement occurred after 24 days post-pollination. Starch from *ae su* differed from all others in that at all ages a large proportion of granules were less than 5 μ in diameter but a few starch granules were almost as large as the largest granules from *normal*. In contrast to *ae wx*, considerable increase was observed between 24 and 36 days post-pollination in the number of *ae su* granules larger than 5 μ.

Possibly, starch granules isolated late in kernel development from physiologically younger cells may reflect the characteristics of starch isolated from the whole endosperm early in kernel development. Gradients of cellular development within the maize kernel have been confirmed by histochemical analyses (1) and by analysis of endosperm zones (4). Further evidence for differences in starch granules has come from reduced incorporation of ^{14}C into starch granules per unit surface area for starch granules containing less than 0.1 γ of starch (3). To test whether smaller starch granules have different amylose percentages when compared to unfractionated starch or larger granules from the same sample, and thus reflect the developmental patterns for amylose accumulations, granules were fractionated by size and apparent amylose percentage measured. Figures 3 and 4 show examples of granules of the size classes separated by dry microsieving and settling through glycerol, respectively.

Smaller starch granules separated from starch of 36-day-old *normal*, *ae-Ref*, and *ae su* kernels might be expected to show a lower amylose percentage than the unfractionated starch, because in all three genotypes, the amylose content of the starches increased with kernel development (Fig. 1). This pattern was observed for the smaller starch granules in all three genotypes (Table II). Starch granules below 5 μ in all three genotypes had amylose percentages near that of unfractionated starch isolated from kernels harvested 24 days post-pollination. Further reduction of starch granule size of *ae-Ref* and *ae su* starch reduced the amylose percentage, but a percentage near that of 18-day starch was not obtained (Table II). However, these starch fractions contained a large proportion of abnormally shaped granules (Fig. 4). The number of abnormal granules increases with development (9,24) and could result from an increasing number of

TABLE I
Number and Average Size of Granules Counted for Starch Granule Size Distributions given in Fig. 2a through 2f

Genotype	Days Post-Pollination					
	18		24		36	
	Number	Diameter[a] μ	Number	Diameter μ	Number	Diameter μ
normal	161,494	3.3	83,671	5.8	80,336	7.7
wx	120,235	3.9	136,792	4.9	256,147	4.1
ae-Ref	53,443	2.8	234,616	3.2	120,555	4.7
ae-il	56,571	3.2	203,244	3.7	169,363	5.1
ae wx	152,642	2.7	313,727	2.9	365,583	3.1
ae su	129,251	2.6	559,722	2.8	182,393	4.5

[a]Average diameter of all particles counted to generate the size distributions. Granule diameter is calculated from the volume measured by the Coulter Counter, assuming that the granules are spheres.

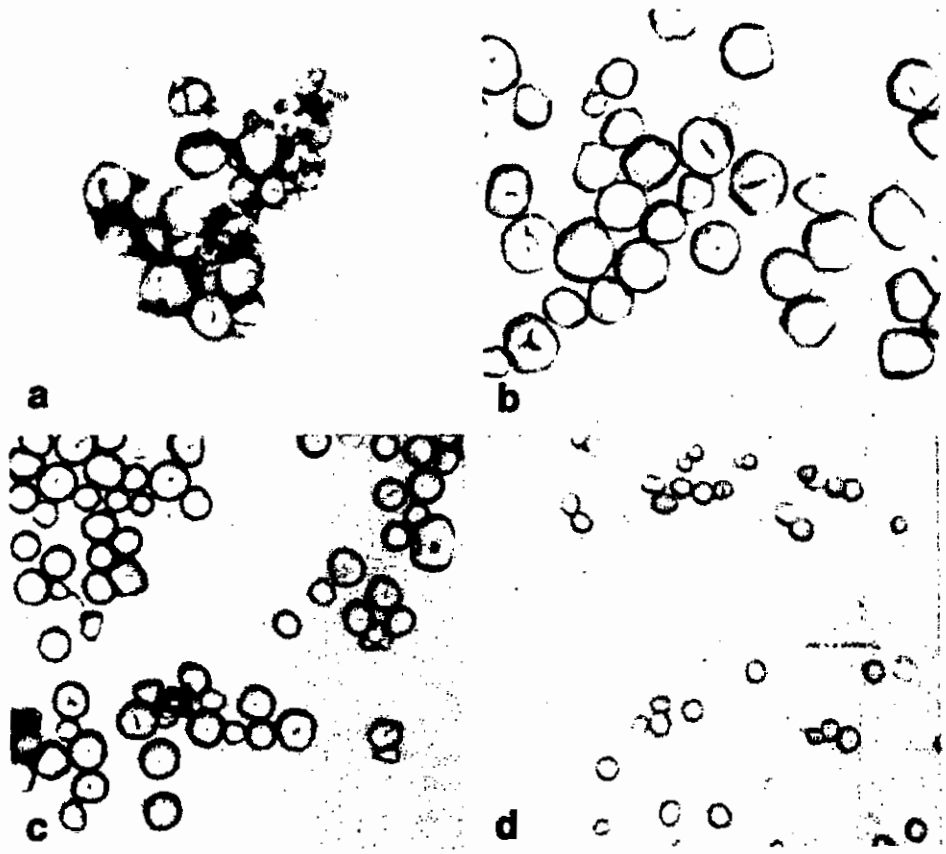

Fig. 3. Examples of *normal* starch granules of different sizes separated by dry microsieving. a) granule clump greater than 20 μ, b) 10 to 20 μ, c) 5 to 10 μ, and d) less than 5 μ. Magnification 256×.

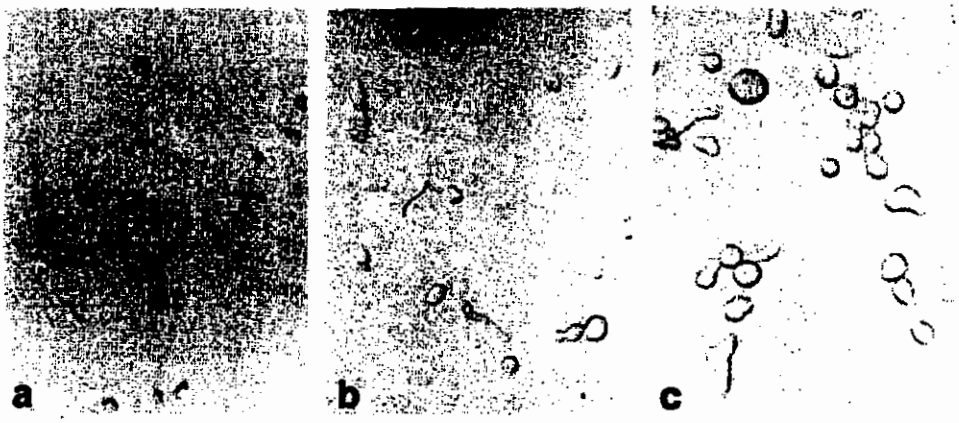

Fig. 4. Examples of small *ae-Ref* starch granules of different sizes separated by settling through 30% glycerol. a) Fraction C-1, b) fraction C-2, and c) fraction C-3. Magnification 256×.

Diameter μ
7.7
4.1
4.7
5.1
3.1
4.5

cells reaching the stage of starch biosynthesis and of *ae* expression when these granule abnormalities are formed. Unpublished observations in our laboratory revealed that the abnormal granules are formed as secondary granules in cells already containing large spherical granules. Since these granules are produced in cells more advanced physiologically than those producing the small spherical granules, the abnormal granules would be expected to have the amylose content reflective of the genotype at a later stage of kernel development. Therefore, the presence of these abnormal granules in the small granule fractions of *ae-Ref* and *ae su* may have kept the amylose content of these starches higher than anticipated. We propose that the smaller spherical granules are indeed derived from physiologically younger cells.

Smaller granules of *ae wx* starch would be expected to be higher in amylose content than the unfractionated starch because the amylose content decreased with kernel development (Fig. 1). No differences in amylose percentages were found among the different size fractions of *ae wx* starch (Table II). Two factors may account for the inability to detect a difference in the amylose content of different sized starch granules from *ae wx* starch. First, only a 10% change in amylose content was observed in starches between 18 and 36 days post-pollination (Fig. 1). In addition, the size distributions generated for *ae wx* starch granules indicated very little change in the size of starch granules between 24 and 36 days post-pollination. Because of these two factors it is not surprising that we failed to detect amylose percentage differences associated with the different granule sizes.

In this study we showed that the amylose percentage increased with increasing age of *normal, ae,* and *ae su* kernels. This increase was due at least in part to an increase in the proportion of larger granules containing a higher amylose percentage. The small spherical starch granules which had lower amylose percentages presumedly were isolated from physiologically younger cells. Tsai *et al.* (5) suggested that there is a shift in starch biosynthetic enzymes in kernels as they begin active starch synthesis, and Shannon (3) proposed that such changes occur in each endosperm cell as it begins active starch synthesis. Therefore, the increased amylose percentage with age is a reflection of the advancing average physiological age of the total endosperm cell population. The single mutant effect and double mutant interactions appear to be expressed at different times in cellular development. Additional study will be necessary to determine what

TABLE II
Amylose Content of Starch Granules of Various Size Classes Isolated from Four Maize Genotypes 36 Days Post-Pollination

Size Class	Genotype			
	ae-Ref	*ae su*	*normal*	*ae wx*
	% of total starch[a]			
Unfractionated	67.8ab	59.0a	25.4a	22.6a
10 to 20 μ	69.5a	61.0a	26.4a	25.6a
5 to 10 μ	64.4b	53.6b	23.0ab	24.1a
Less than 5 μ	58.3c	47.0cd	20.5b	25.4a
C-3	63.8b	48.4c	...	...
C-2	57.3cd	43.6cd	...	...
C-1	53.0d	43.3d	...	...

[a]Values followed by the same letter within a column are not significantly different ($k = 100$).

enzyme changes are responsible for the mutant effects on starch composition.

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